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The goal of this study has been to test the hypothesis that the *Notch* gene controls axon extension in *Drosophila* by a mechanism distinct from that by which it controls cell fate, and if so, to identify *Notch* domains and downstream effectors that execute this function. This analysis has involved investigation of the phenotypes of *Notch* mutants and their interactions with mutations in genes encoding other signaling proteins, expression of *Notch* derivatives *in vivo* in both wild type and mutant genetic backgrounds, and examination of the biochemical interactions between Notch and other signaling proteins *in vitro*. Our experiments have established that the function of *Notch* in axon patterning is indeed genetically separate from the *Notch*-dependent control of cell fate; that the axonal function of *Notch* requires tethering of the protein to the plasma membrane; and that the *abl/disabled* signaling pathway executes the axonal function of *Notch*, perhaps *via* interaction with small GTPases of the Rho subfamily. In the course of these studies, we have also demonstrated directly that *Notch* can arrest cells at a precursor stage of their development and have observed indications that the *Notch/abl* pathway may play a role in genetic instability.

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5. INTRODUCTION

A. Subject, purpose and scope of this study

Notch is an oncogene that is implicated in the development of hematologic cancers and breast cancer in mammals, including humans (1-5). In order to understand the mechanism of oncogenesis by *Notch*, and as a basis for investigating potential therapeutic strategies, a great deal of effort has been devoted to characterizing the biological functions of Notch protein and the signaling pathways by which it performs those functions.

The eponymous founding member of the *Notch* family of proteins is a developmental control protein of *Drosophila melanogaster* (6, 7). It is in this organism that we know the most about the activities of *Notch in vivo*, and that we have the most powerful set of genetic and molecular reagents for assaying and manipulating these activities. The purpose of the study reported here was to exploit the *Drosophila* system to extend our understanding of the activities and signaling mechanisms of *Notch*. In particular, our goal was to test the hypothesis that *Notch* can directly control cell morphology and invasiveness by a mechanism distinct from that by which it controls cell identity. To accomplish this, our approach was, first, to further characterize developmental activities of *Notch* in *Drosophila* that are (plausibly) related to its actions as an oncogene in mammals, second to identify domains of *Notch* that are specific for its different activities, focusing on those that are specific for the control of cell morphology and invasiveness, and third, to identify downstream proteins that transduce these different *Notch* signals, again focusing on the control of cell morphology and invasiveness. In these studies, we analyzed the effect of *Notch* on the growth of axonal projections in the developing nervous system as an assay for activity in morphogenesis and invasiveness, and we analyzed neurogenesis, myogenesis and oogenesis as assays for the *Notch*-dependent control of cell fate.

We will now describe the background of previous work underlying the experiments we performed. In particular, we will describe what was known prior to this study about the activities and signaling pathways of *Notch*, and we will describe a signaling pathway, the Abl genetic pathway, that has previously been implicated in axon outgrowth in *Drosophila* and which we identified in the course of our work as one signaling pathway by which *Notch* apparently controls cell morphology and invasiveness.

B. Background of previous studies.

Notch: Activities and signaling pathways.

In *Drosophila*, *Notch* is required for the proper specification of cell identities at all stages in development, and in nearly every tissue in the animal. *Notch* has been shown to act in two kinds of cell fate decisions. The first of these is a process termed "lateral signaling", in which a cluster of cells start out with equivalent developmental potential and one of these cells is singled-out to take on a distinct and different identity (8). For example, in the developing neuroectoderm, an entire "proneural cluster" has the potential either to remain as epidermal cells or to become neuronal precursors (neuroblasts). In wild type embryos, only one cell in the proneural cluster becomes a neuroblast, whereas in the absence of *Notch* function, *all* the cells in a cluster become neuroblasts. This suggests that the role of *Notch* is to restrict the developmental potential of most of the cells in the equivalence group, and permit only one cell to enter the alternate developmental pathway, in this case, neurogenesis. *Notch* is also required for a second kind of cell fate decision, that by which two sibling cells in a lineage reproducibly choose to take on different developmental identities (9, 10). For example, in a developing sense organ a single precursor cell divides to generate two daughters, an "inner cell" that will migrate under the epithelium and give rise to a neuron and a glial cell, and an "outer cell" that will remain in the epidermis and give rise to two mechanosensory support cells -- a bristle cell and its surrounding socket (Fig 1A). *Notch* is

normally active only in the "outer" daughter cell. In the absence of *Notch* function, the two daughters of the sense organ precursor both become "inner cells", while if *Notch* is hyperactivated, both daughters of the sense organ precursor become "outer cells". Thus, *Notch* is the switch which instructs these two sibling cells, the "inner" and "outer" cells, to take on different identities (11). Throughout development, the role of *Notch* in every example of lateral signaling that has been examined seems to be analogous, and the same is true for every known example of sibling cell signaling.

Notch encodes a transmembrane receptor protein that appears to use a single, common signal transduction pathway to control cell fate in the vast majority of developmental contexts in which it has been studied (12, 13). Upon binding to ligand (in *Drosophila*, most often the Delta protein), Notch is thought to be proteolytically cleaved at the inner surface of the plasma membrane and nucleate the formation of a multiprotein complex that includes the transcription factor Su(H), and probably other proteins as well (14-16). This complex then translocates to the nucleus, where it binds to target genes that bear Su(H) recognition sites in their promoters, activating their transcription and thereby controlling cell phenotype. This same basic mechanism seems to obtain both in lateral signaling and in the control of sibling cell identities. The two types of *Notch* signaling are distinguished, however, by differences in the auxiliary proteins that assist in each process. For example, Neuralized and the proteins encoded by the *E(spl) Complex* are absolutely required for lateral signaling but seem not to be involved in sibling cell signaling, whereas Spdo and Numb both are involved in sibling cell signaling but apparently are dispensable for lateral signaling (9, 10).

In *Drosophila*, we know of only a few *Notch*-dependent cell fate decisions that are not explained by the mechanism just described (17-19). For example, within the adult sense organ lineage, once the inner precursor cell is generated (by a mechanism requiring an appropriate pattern of *Notch* function, see above) this cell divides again to produce a neuron and a glial cell. Specification of cell fates following this second division is also dependent on *Notch*: in the absence of *Notch*, both daughters of the inner precursor become neurons while hyperactivation of *Notch* causes both daughters to differentiate as glial cells. However, it has been demonstrated that this function of *Notch* does *not* involve the usual *Notch* effector, *Su(H)*, even though *Su(H) is* required both for the inner vs outer cell fate decision, and for the analogous decision between bristle and socket fates of the progeny of the outer precursor (18). Similarly, in the early embryo, specification of midline cells requires *Notch* but is independent of *Su(H)* (though it is not clear what alternate fate is assumed by cells that fail to become midline cells in *Notch* mutant embryos).

In addition to the control of cell fate by *Notch*, there is evidence that *Notch* is required for the growth and guidance of specific axons in the developing *Drosophila* nervous system (20). In the absence of *Notch* function, axons in the central nervous system (CNS) fail to grow longitudinally from one segment to the next, and axons of the peripheral nervous system (PNS) fail to grow dorsoventrally along a particular cellular substratum, the lateral peritracheal cells. The initial studies hinted that this effect of *Notch* on axon patterning might not be secondary to errors in cell identity, but might actually reflect a direct function of *Notch* in axons.

The role of *abl* and *abl*-interacting genes in axon outgrowth

Abl is a cytoplasmic protein tyrosine kinase which, like *Notch*, seems to be conserved throughout metazoan development. Also like *Notch*, *abl* is an oncogene that is strongly implicated in particular hematologic malignancies, and so has been studied intensively for many years (21). Unlike *Notch*, however, it has been very difficult to link Abl convincingly to any receptor or extracellular signal in normal development. The signaling mechanism downstream of Abl also remains obscure, though it appears that small GTPases of the Rho subfamily -- Rac and Cdc42 -- are likely to be somehow involved (22-24). Rho family GTPases are implicated both in control of nuclear

gene regulation via the MEKK/Jnk MAPK cascade, and also in direct control of the actomyosin cytoskeleton (25).

The clearest evidence for the *in vivo* function of *abl* and the genes through which it operates comes again from genetic studies in *Drosophila* (26-28). Abl is expressed ubiquitously in *Drosophila*, with the highest protein concentration found in axons of the nervous system, and in muscles where they attach to the epidermis. Mutant animals lacking *abl* are largely viable, though they display defects in eye development and mutant females are essentially sterile. Presumably, the survival of *abl* mutant animals implies that the action of *abl* is redundant with that of other proteins, though these other proteins have not yet been identified. A genetic screen was performed some years ago to isolate genes which are haploinsufficient in the background of a homozygous *abl* mutation, under the hypothesis that this would identify genes which collaborate with *abl* in its normal signaling pathway. Three genes were identified by this approach, called *disabled, fax* and *prospero* (collectively termed the *HDA* loci, for *haploinsufficient, dependent on abl*) (27-29). Consistent with the *abl* expression pattern, it was found that combining *abl* and *HDA* mutations caused severe defects in axon extension, and dramatically weakened the attachment of muscles to the epidermis. Moreover, the localization of the HDA proteins was also consistent with a connection to *abl* signaling in that all three of these proteins were found to be concentrated in axons. The functions of the HDA proteins themselves remains somewhat less clear. Disabled (Dab) appears to be an adaptor protein, with multiple protein-protein interaction motifs; Fax is a ser, thr protein kinase and Pros is required for axons to extend at the proper time (in addition to acting earlier in embyrogenesis as a developmental determinant that is localized to one portion of the cortical cytoplasm in dividing neuroblasts and then translocates to the nucleus in second-order neuronal precursors) (28-32) The precise roles of these proteins in axonogenesis, however, and specifically how they cooperate with one another and with Abl to comprise an axonal signaling pathway, remains mysterious.

6. BODY

A. Methods

Drosophila genetics and immunocytochemistry

Flies were maintained by standard methods. All mutations are described in Lindsley and Zimm (33). *UAS-Notch* was obtained from Gerold Schubiger; *elav-GAL4, UAS-rac^{G12V},UAS-rac^{S89L}* and genetically-marked Y chromosomes were obtained from Y.N. Jan; a *lacZ*-marked balancer for the second chromosome was obtained from Corey Goodman; and compound autosome stocks were obtained from the *Drosophila* Genetics Stock Center. All expression of transgenes *in vivo* in flies employed the GAL4-UAS$_G$ *Drosophila* expression system (34, 35).

The protocol for *Notch^{ts}* temperature-shift experiments was as follows: embryos were collected at 18^o for 6 hours, aged an additional 6 hours at 18^o, shifted to 32^o for 7.5 hours and then fixed. This timing was designed to produce highly-penetrant axonal phenotypes while minimizing neurogenic defects, taking into account the phenotypic lag between the time of the temperature-shift and the decay of activity of Notch^{ts1} mutant protein (20, 36). Embryo fixation and immunocytochemistry was by standard methods; except embryos to be stained with anti-Odd were pretreated with 9:1 MeOH:H_2O_2 for 15 minutes at room temperature, and then washed three times with 0.1 M NaPhosphate pH 7.2; 0.3% TritonX-100, prior to blocking. Antibodies used were: anti-Fasciclin 2 (1:375; Corey Goodman), anti-Odd (1:6000; Ellen Ward), mAb22C10 (1:375; Hugo Bellen) and anti-β-gal (1:10,000; Cappel).

The protocol for expression and analysis of transgenes in adult sense organ precursors was as follows: flies bearing a UAS-*Notch$^{\Delta E}$* or UAS-*rac* transgene were crossed to GAL4 line 109-68

(Ref 18) at 18°. G4 109-68 is expressed in all the cells of the adult sense organ lineage. Progeny bearing both P-elements were collected as white prepupae and incubated 24-30h at 25°. Pupae were then fixed and stained with antibodies by standard methods (18), with visualization of the antibody signal by peroxidase histochemistry. Antibodies used were: mAb22C10 (1:375; Hugo Bellen) or rat anti-Elav (1:20; Developmental Hybridoma Stock Center) to label neurons, and mAb MR1a (anti-Prospero; 1:50; Chris Doe) to label glia. After staining, pupal nota were dissected, mounted in 50% glycerol and examined by Nomarski microscopy.

in vitro differentiation and culture of *Drosophila* primary embryonic neurons

in vitro culture of primary *Drosophila* neurons was done as decribed by Spana and Doe (9); fixation and antibody staining of cultured cells was by standard methods. The growth cone marker for this experiment was a kinesin-β-galactosidase fusion protein, expressed by enhancer trap line KZ-636 (37). Samples were mounted in FluoroGuard (BioRad) and examined with a BioRad MRC600 confocal microscope. Authenticity of the fluorescent signals was verified by omitting or replacing the primary antibody, and by separate, sequential excitation of the two fluorophores in double-label experiments. Antibodies used were: mAb17.9C6 (anti-Notch; 1:1000; Mann and Artavanis-Tsakonas) and anti-β-galactosidase (1:10,000; Cappel).

Binding of Notch and Disabled *in vitro*

1. Expression of Disabled and Disabled derivatives: ^{35}S-labelled Disabled protein was expressed by coupled transcription/translation *in vitro* in a reticulocyte lysate following the manufacturer's recommended protocol (TNT system; Promega). Full-length Disabled was expressed from a clone in pBluescript provided by Allen Comer and Mike Hoffmann; the PTB domain alone was expressed by linearizing the Disabled clone with PvuII (which cuts ~20 codons 3' to the end of the PTB domain) prior to performing the transcription/translation reaction. A Disabled derivative lacking the PTB domain (Δ-PTB Disabled) was prepared by subcloning the 5' EcoRI fragment of Disabled into pBluescript, performing site directed mutagenesis (ExSite mutagenesis system; Stratagene; per manufacturer's protocol), and reconstructing the deleted EcoRI fragment back into the context of the full-length gene. For the site directed mutagenesis, deoxyoligonucleotides were designed to delete codons Asn^{39} - Ile^{180} (inclusive). Expression of Δ-PTB Disabled by *in vitro* transcription/translation was done precisely as for the full-length gene.

2. Preparation of beads bearing portions of the *Notch* intracellular domain: Construction of plasmids expressing His$_6$-tagged derivatives of various portions of the *Notch* intracellular domain was described in our Annual Report of 10/97. His-tagged proteins were purified from bacteria essentially as described for purification of the His-tagged complete *Notch* intracellular domain (Annual Report, 10/97), except the dialysis of proteins following purification on a Ni^{+2} column was into a buffer appropriate for coupling to CNBr-activated Sepharose (100mM $NaHCO_3$, pH8.3; 100mM NaCl; 0.25% (v:v) Triton X-100; 0.1 mM PMSF). Following purification, proteins were clarified by centrifugation at 8 Krpm for 12 minutes. The soluble, purified protein was then coupled to CNBr-activated Sepharose 4B (Pharmacia) by the manufacturer's protocol.

3. Binding experiments: Protein binding studies were performed precisely as described in our Annual Report of 10/97, except the solid phase proteins were the Sepharose-coupled Notch derivatives and the solution phase proteins were the *in vitro* translated Disabled derivatives. Bindings were done in the standard binding buffer described previously, or in the same buffer with 1% NP40 and 0.1% SDS.

B. Results and Discussion

Rationale of the project

Experiments we performed prior to the initiation of this study had led to the hypothesis that the activity of *Notch* as an oncogene reflects its ability both to control cell identity and also to directly control cell morphology and invasiveness. The experiments in this project were designed to test that hypothesis, exploiting the *Drosophila* embryo as a system to study *Notch* functions, and in particular using extension of axons during neuronal development as a surrogate assay for cellular morphogenesis and invasiveness. The goals of the project were first to characterize actions of *Notch* in the fly that plausibly might reflect functions of the protein that are relevant to its oncogenic potential, and to develop reagents that permit us to dissect and manipulate these activities, and then to identify signaling domains of *Notch*, as well as the signaling proteins that work downstream, discriminating in particular the pathway that controls axon outgrowth from that controlling cell identity.

We will now describe in turn how we have approached and acheived each of these goals.

Activities of *Notch* in *Drosophila*

1. Control of cell identity in adult sense organs: Characterization of an activated derivative of *Notch*

Prior to the start of this project, we constructed a truncated derivative of *Notch* lacking the extracellular domain, called $Notch^{\Delta E}$. Preliminary experiments suggested that this derivative was likely to be constitutively activated, as it gave rise to phenotypes that seemed "opposite" to known phenotypes of *Notch* loss-of-function mutants. For example, whereas a *Notch* mutation leads to hyperplasia of the nervous system due primarily to segregation of extra neuronal precursors, we found that expression of $Notch^{\Delta E}$ in the developing neuroectoderm produced neural hypoplasia, with reduced numbers of neuroblasts and differentiated neurons (51, and EG, unpublished observations). To confirm that this indeed reflects a reversal of the *Notch* phenotype we investigated the effect of expressing $Notch^{\Delta E}$ in developing adult sense organs, where *Notch* is required to specify the identities of both progeny of the initial precursor cell, and to specify the identities of all four of the cells of the mature sense organ. If $Notch^{\Delta E}$ is indeed a constitutively-active derivative of *Notch*, then expression of this protein throughout the lineage would be predicted to yield a sense organ with no inner cells (glia or neurons, detected, respectively, with mAb MR1A, and with either mAb22C10 or anti-Elav), and no bristle, but only four socket cells. Alternatively, expression of $Notch^{\Delta E}$ just in the outer precursor would give an organ with two sockets and no bristle, while expression just in the inner precursor would give rise to an organ with two glia but no neuron. This constellation of phenotypes was indeed precisely what we observed upon expression of $Notch^{\Delta E}$ in the sense organ lineage (Fig 1), providing strong evidence that $Notch^{\Delta E}$ constitutes an activated derivative of *Drosophila Notch.*

1.B. mouse *Notch1* provides *Notch* activity in *Drosophila*: Evidence that mouse *Notch1* is a true homolog of *Drosophila Notch*

The rationale behind this project assumes that Notch-like proteins of mammals are true homologs of *Drosophila Notch*, that is, that they use a similar mechanism to perform equivalent functions. To test this assumption, we collaborated with R. Kopan and H. Weintraub to prepare derivatives of mouse *Notch1* that were analogous to activated derivatives of *Drosophila Notch* prepared by us and by others, and we expressed these modified proteins in the fly. As documented in Fig 5B of our Annual Report of 10/95, we found that the intracellular domain of mouse *Notch1* could provide *Notch* activity in *Drosophila*. For example, upon expression of activated m*Notch1* in developing adult *Drosophila* sense organs we observed two-socket and four-socket phenotypes that were indistinguishable from those induced by expression of the *Drosophila* protein. The finding that mammalian and fly *Notch* are true homologs provided important justification for our experimental approach, arguing that what we learn about the fly protein will be applicable to the mammalian protein as well. Evidence obtained independently by other labs over the past 4 years has further supported this conclusion by demonstrating directly that vertebrate *Notch* homologs

signal *in vivo* via proteins that are similar in their sequence and in their properties to the proteins known to act downstream of *Notch* in *Drosophila* (14, 38).

2. Multiple roles of *Notch* in *Drosophila* myogenesis

> Studies in this section were documented in our Annual Report of 10/96, and in a published manuscript (39).

In order to further verify that $Notch^{\Delta E}$ was indeed a constitutively activated derivative of *Notch* we next investigated its activity in a second, independent developmental context. Muscle precursor cells in *Drosophila* are specified in a manner that is in some ways analogous to the specification of neuronal precursors. First, multipotent progenitor cells segregate from among a cluster of equivalent myogenic cells, much as neuroblasts segregate from proneural clusters of neuroepithelial cells (40). Next, each progenitor cell develops along an invariant lineage to give rise to the founder cells for particular muscles, much as each neuroblast or sense organ precursor follows a specific lineage to give rise to particular neurons and neural support cells (41). Previous studies had implied that the first step in myogenesis, selection of a single progenitor cell from a myogenic cluster, proceeded by a process of *Notch*-dependent lateral signaling (42). We verified this inference by using $Notch^{\Delta E}$ to activate *Notch* signaling in myogenic territories and found that, as expected, this blocked the segregation of muscle progenitors (Ann. Rep. 10/96, Fig 2 A,B).

Our experiments also uncovered a second function of *Notch* in myogenesis. In a significant fraction of embryos, myogenic progenitors escaped from the initial effects of $Notch^{\Delta E}$, particularly if the protein was expressed at relatively low levels. When this occurred, we found that expression of $Notch^{\Delta E}$ in developing myocytes could also affect muscle development at a later stage, during the evolution of each progenitor lineage to produce particular founder cells. Specifically, we found that the expression of multiple founder cell markers (Vg, S-59) was extinguished late in development by expression of $Notch^{\Delta E}$ as assayed by immunocytochemistry, and that apparently as a consequence, the founders never underwent morphogenesis into elongated fibers, and never recruited surrounding myoblasts into multinucleate myotubes (Ann. Rep. 10/96, Fig 2 C-E). To verify that this phenotype reflects a function of *Notch* in normal myogenesis and not a neomorphic effect of the activated protein, we investigated the effects of late temperature-shifts of a temperature-sensitive mutant allele of *Notch*. Consistent with the phenotypes observed upon expressing the activated protein, we found that shifts of $Notch^{ts1}$ embryos to the nonpermissive temperature subsequent to progenitor segregation nonetheless led to hyperplasia of founder cells as assayed by immunocytochemistry with a founder marker (S-59); ie, it induced a phenotype opposite to that of $Notch^{\Delta E}$.

Two models are consistent with our observations of a second function of *Notch* in myogenesis. It could be that *Notch* acts at each division in every progenitor lineage to discriminate the identities of the two sibling progeny cells, much as it does in sense organ lineages. This model is suggested by the observation that, in some lineages, manipulation of the *Notch* antagonist *numb* can phenocopy the effects we observe from manipulating *Notch* activity (41). Alternatively, or additionally, it may be that the decision of a cell to be a progenitor/founder cell or to remain an uncommitted fusible myoblast remains plastic to *Notch* activity for a period of time after progenitor segregation, much as the neural/epidermal decision in proneural clusters is thought to remain plastic to *Notch* activity for some time after neuroblast segregation. This possibility is suggested by the observation that a *numb* mutation appears only to affect a fraction of the myogenic lineages which are sensitive to late activation of *Notch* signaling (41). Discriminating between these models will require additional analysis of the identities of cells in specific myogenic lineages, both in wild type animals and in animals with increased or decreased levels of *Notch* signaling.

2. B. Non-autonomous inhibition of myogenic differentiation by ectodermal *Notch*

It has typically been assumed that the effects of *Notch* mutations on cell specification during myogenesis reflect autonomous functions of *Notch* protein in the myogenic cells themselves. Experiments described above using $Notch^{\Delta E}$ verify that *Notch* can indeed produce these effects cell-autonomously, since an appropriate promoter system was employed in these experiments to ensure that the modified *Notch* derivative was expressed only in myogenic cells. However, these results do not rule out the possibility of a separate, non-autonomous *Notch*-dependent effect on myogenesis, mediated by control of some inductive influence from the ectoderm. Such a notion is suggested by a variety of experiments from others which indicate that the ectoderm can influence muscle development in *Drosophila* (43, 44).

To examine possible effects of ectodermal *Notch* signaling on myogenesis, we expressed $Notch^{\Delta E}$ solely in the ectoderm of otherwise wild type fly embryos and analyzed muscle development. To our surprise, we found that activation of *Notch* signaling in the ectoderm seriously disturbed myogenesis, with most muscles failing to elongate and fuse, even though ectodermal differentiation and morphogenesis was essentially unaffected by this protocol. We also found that myoblast expression of founder cell markers (S-59 and Vg) was extinguished by activation of *Notch* signaling in the ectoderm, though whether this was the cause or the consequence of the failure of muscle differentiation could not be distinguished (Ann. Rep. 10/96, Fig 2 F-H). Since the activated *Notch* derivative we were expressing contained no extracellular *Notch* sequences, the effect of *Notch* necessarily must reflect its influence on some signaling pathway within epidermal cells, and not a direct effect of the *Notch* extracellular domain on underlying myocytes.

Evidently, *Notch* signaling in the epidermis either induces the expression of some non-autonomous inhibitor of myogenesis, or it inhibits the expression of some required activator of muscle development. It is not yet clear whether this observation reveals a novel function of wild type *Notch* in normal development or whether it is a neomorphic effect of the activated protein. We note, however, that the timing of overt muscle differentiation in wild type embryos, and the time at which artificial *Notch* activation in the ectoderm leads to myogenic defects, correlates closely with the time when widespread expression of the *Notch* ligand, Delta, decays from the ectoderm in wild type embryos (45; S. Fuerstenberg and EG, unpublished obs). It may be that *Delta* expression, and thus *Notch* activity, in the epidermis sets the timing of muscle differentiation in wild type animals, thereby temporally coordinating mesodermal differentiation with ectodermal differentiation.

We do not yet know the identity of the *Notch*-regulated non-autonomous signal by which the epidermis controls the development of underlying muscles. We note, however, that it has recently been shown that myogenic expression of a dominant-negative FGF receptor late in muscle development leads to phenotypes that closely resemble those we observed from activation of *Notch* (46), suggesting that an FGF-like molecule may be a good candidate for the signal that is regulated by *Notch* in our experiments. If so, this would be of general interest for understanding vertebrate development since the *Notch* and FGF pathways intersect in a number of other developmental contexts, such as growth and patterning of the vertebrate limbs. Moreover, regulation of FGF signaling by *Notch* would be of particular interest in the context of breast cancer. In development of the breast, it is thought that secretion of FGF by epithelial cells controls the proliferation and differentiation of nearby mesenchymal cells, much as secretion of FGF by embryonic epithelial cells of *Drosophila* is thought to control the differentiation of underlying mesodermal cells. Moreover, in the breast, the FGF signal feeds back on the epithelial cells by modulating the secretion of TGF-β by the mesenchymal cells (47-49). If *Notch* controls FGF production by epithelial cells of the mammalian breast, as we propose for the *Drosophila* epidermis, this would provide an additional, non-autonomous mechanism by which dysregulation of *Notch* might contribute to the development of epithelial tumors of the breast.

3. *Notch* activity arrests cells at a precursor stage of development: Evidence from oogenesis

Analysis of *Notch* loss-of-function mutants indicated that *Notch* is required at multiple steps in the specification and differentiation of ovarian follicle cells during oogenesis (50). In collaboration with the laboratory of H. Ruohola-Baker, we exploited the activated *Notch* derivative $Notch^{\Delta E}$ described above to further investigate the roles of *Notch* in oogenesis. Specifically, we artificially activated *Notch* signaling in follicle cells to varying degrees and at various times during oogenesis by expression of this truncated protein. As documented in our Annual Report of 10/96 and in a published manuscript (51), we verified that *Notch* acts at multiple steps in follicle cell development to specify sequential cell fate decisions during differentiation: expression of $Notch^{\Delta E}$ early in follicle cell development transformed presumptive polar cell precursors into extra stalk cells, while expression later in development transformed polar cells into extra flanking cells (Ann. Rep. 10/96, Fig 1 A-D). Both of these phenotypes are opposite to the phenotypes induced by manipulations which decrease *Notch* signaling at these stages of development. In the course of these studies, we also obtained direct evidence that activation of *Notch* signaling arrests cells in a precursor state of development: analysis of the marker protein Bib showed that the extra stalk cells induced by activation of *Notch* had the position, morphology and polarization appropriate to stalk precursor cells rather than to mature stalk cells (Ann. Rep. 10/96, Fig 1 E-G).

The finding that *Notch* arrests ovarian follicular stalk cells at a precursor stage of development is of great significance for understanding the oncogenic effects of *Notch*. Many investigators have proposed that *Notch* acts in a variety of developmental contexts to inhibit differentiation, that is, to maintain cells in a precursor stage of development. Precursor-type cells are typically plastic in their identity, are often more motile than differentiated cells and often are still capable of proliferating. It has therefore been suggested that one important way that activation of *Notch* promotes oncogenesis is by diverting cells into a proliferative and invasive cellular compartment, that is, by locking them into a precursor state. The gap in this argument, however, has been the failure to observe positive evidence that the developmental state induced by *Notch* is indeed a precursor state: there have been numerous examples of failure to observe the expression of specific differentiation markers in cells in which the *Notch* pathway is activated, but in these cases it had not been shown that the developmental state conferred was indeed a precursor state rather than simply an alternate mature, differentiated state. Our studies of oogenesis afforded the first published example in which markers were available to demonstrate directly that the effect produce by *Notch* was that of stably locking cells into an immature, proliferative precursor state: the pre-stalk identity.

4. *Notch* is localized to growth cones and is required at the time of axon outgrowth for proper axon patterning

Previous experiments from our lab had shown that two specific axon guidance decisions in the developing fly embryo are disturbed in *Notch* mutants, but these experiments could not distinguish whether this reflected a direct role for *Notch* in axons or an indirect effect mediated by *Notch*-dependent control of cell fate (20). Our initial studies employed temperature shifts of a temperature sensitive mutant of *Notch*. The interpretation of these experiments, however, was complicated by the fact that for a given neuron the window between the time of identity specification and that of axon outgrowth is very short in the fly embryo, and indeed, pioneer axons begin to grow while other neurons are still being born. Thus, it is not possible simply to use temperature shifts of a $Notch^{ts}$ mutant to cleanly separate effects of *Notch* on neuronal identity from effects on axon growth and patterning.

As an alternate approach to separating the axonal function of *Notch* from the control of cell fate, we combined the use of a temperature-sensitive *Notch* mutation with a promoter system that permits precise temporal and spatial control of the expression of a wild type *Notch* transgene. Specifically,

into the background of *Notch^{ts1}*, we crossed a pair of transgenes that lead to expression of wild type *Notch* only in postmitotic neurons late in differentiation; ie after their identities have been specified but at about the time of axon outgrowth. We already know that raising a *Notch^{ts1}* embryo to restrictive temperature at an appropriate time prevents the growth of specific axons, as discussed above. Upon adding back wild type *Notch* only in differentiated neurons we found that axonal defects were almost completely rescued (>85%; Fig 2 A,B), even though those low-level defects that do occur due to the temperature shift are unaffected by this late expression of wild type *Notch* (Fig 2, C-E). Thus, this experiment shows directly that *Notch* is required at the time of axon outgrowth for proper axon patterning to occur, and that the cell fate changes which are induced as a byproduct of our standard *Notch^{ts}* temperature-shift protocol are not sufficient to cause the observed defects in axon patterning. In conjunction with our finding that *Notch* can produce axonal defects without detectable alteration of neuronal identities (in conjunction with *abl*; data obtained in experiments funded by the March of Dimes Birth Defects Foundation, discussed below and in our DOD Annual Report of 10/96), these data complete the formal demonstration that the axonal function of *Notch* is genetically separable from its role controlling neuronal cell fate.

The hypothesis that *Notch* is required directly in the axon for axon extension makes a strong prediction that the protein should be present in the growth cones of extending axons. This is difficult to test *in situ* since *Notch* is expressed ubiquitously, including in the substratum cells on which these axons extend. We therefore turned to a simplified *in vitro* culture system to investigate the subcellular localization of Notch protein. Early stage embryos (4-8 hours after egg laying) were mechanically dissociated to single cells by homogenization, the cells were washed with culture media, plated on glass coverslips and allowed to grow and differentiate overnight. Cells were then fixed with formaldehyde and Notch localization was assayed by indirect immunofluorescence with anti-Notch antibodies (Fig 3). Notch protein was observed at the tips of growing axons, in bulbous, flat and spiked structures that resembled growth cones. The identification of these structures as bona fide growth cones was verified by co-staining with a characterized growth cone marker (a fusion protein linking *E. coli* β-galactosidase to the motor domain of *Drosophila* kinesin, expressed in neurons (37)). Thus, Notch protein is indeed found in growth cones, as expected if it contributes directly to axon growth and guidance.

Data in this section were reported in a published manuscript (52).

Signaling domains and downstream effectors.

Experiments presented above characterized *in vivo* functions of Notch protein, and established a set of reagents and methodologies for assaying and manipulating those functions. In particular, these experiments argue strongly that *Notch* plays a role in axon outgrowth that is separate from its function in control of cell fate, thereby validating the central hypothesis of this study. Below we present experiments that investigate this novel function of *Notch*, in particular by identifying domains of *Notch*, as well as downstream signaling proteins, that execute the *Notch*-dependent control of axon outgrowth.

1. Genetic interaction of *Notch* and *abl*

As described in the Introduction, Abl is a cytoplasmic protein tyrosine kinase which in *Drosophila* is concentrated in axons and is required for axon extension. Due to genetic redundancy, however, the axonal requirement for *abl* can only be observed in embryos that are also partially deficient in one of the genes that collaborate with *abl* (called "*HDA* loci"), such as the gene encoding the adaptor protein Disabled. In the context of experiments funded by the March of Dimes, we found that *Notch* has the properties of an *HDA* locus: embryos that are heterozygous for a *Notch* mutation and homozygous for an *abl* mutation show severe and specific defects in the extension of *Notch*-dependent axons. Importantly, we also found that these axonal defects occurred in the

absence of detectable alterations in cell identity, suggesting that this novel *Notch/abl* pathway might be the mechanism by which *Notch* directly controls axon outgrowth.

2. Physical association of Notch and Disabled

If the *abl* signaling pathway executes the axonal function of *Notch* it should be possible to identify a physical association between the Notch intracellular domain and one of the Abl accessory proteins. We noted that the Disabled protein includes a protein interaction domain, called a PTB domain, that is closely related to the Notch-binding domain of the Notch interacting protein Numb. We therefore investigated the possibility that Disabled (Dab) might bind directly to Notch. In a published manuscipt (52), and in our Annual Report of 10/97 we reported three experiments which indicated that the Dab PTB domain can bind to the Notch intracellular domain. First, a fusion of the Disabled PTB domain to GST selects Notch out of a total embryo lysate *in vitro* (Ann. Rep. 10/97, Fig 1). Second, Dab(PTB)-GST also binds Notch when both proteins are purified from *E. coli,* showing that the interaction is direct (Ann. Rep. 10/97, Fig 3A). Third, the Dab(PTB)-GST binds only one signaling domain of Notch, the amino-terminal "Ram23" domain and not the ankyrin repeats, opa region or notchoid region, showing that the interaction is selective and specific (Ann. Rep. 10/97, Fig 2). (Note: When we initially reported the *in vitro* binding of Notch and Disabled in our Annual Report, it appeared that our data were potentially consistent with modulation of binding strength by ser, thr phosphorylation of Notch. Further investigation suggests that this effect is assay dependent and is more likely to reflects some aspect of the experimental protocol than of *Notch* regulation (data not shown).)

The experiments reported in our Annual Report of 10/97 and in (52) failed, however, to address several important questions about the physical interaction of Notch with Disabled. First, all the binding experiments investigated the association of bead-bound Disabled with soluble Notch. The evidence would be more compelling if the association can also be detected when performed in the opposite way, with soluble Disabled and bead-bound Notch. Second, we showed that the Dab PTB domain is sufficient for binding to Notch but not that it is necessary. In principle, there could also be interactions between Notch and other domains of Disabled.

We have now demonstrated that beads bearing Notch protein can bind soluble Disabled protein *in vitro*. A His_6-tagged derivative of the *Notch* Ram23 domain was purified from bacteria and coupled to CNBr-activated Sepharose beads. Upon mixing these beads with either full-length ^{35}S-labelled, *in vitro*-translated Disabled (Fig 4A), or Disabled PTB domain (Fig 4B), we found that the Ram23 beads efficiently precipitated the Disabled protein, whereas beads bearing, for example, the Notch ankyrin repeats did not. This interaction occurs even in the presence of 1% NP40 and 0.1% SDS (not shown), suggesting that it is likely to reflect an authentic binding interaction. We also used this *in vitro* system to begin investigating whether there might be additional sites of interaction between Notch and Disabled. We used site-directed mutagenesis to completely delete sequences encoding the PTB domain of Disabled. We then prepared the Δ-PTB protein by *in vitro* translation and assayed its binding to beads bearing various signaling domains of Notch. To our surprise, we found that there was significant residual binding of Δ-PTB Dab to Notch, particularly the Ram23 domain (Fig 4C). Though this association was less efficient than the binding of the full-length Dab protein, it was nonetheless readily detectable even under highly stringent binding conditions of 1% NP40 and 0.1% SDS. This observation raises the possibility that the interaction of Notch with Disabled is bidentate which, if true, would greatly increase its avidity. To provide the background necessary to design tests of the significance of these interactions *in vivo*, it will be important to determine whether the interaction is direct, to map the second putative Notch binding domain on Dab, and to measure the absolute affinities of the two interactions in order to determine which is potentially relevant *in vivo*.

3. Discriminating *Notch* domains required for axon outgrowth from those which control cell fate: Notch must be tethered to the plasma membrane to promote axon extension

We demonstrated above that we can specifically assay the axonal function of *Notch* by expressing the gene from an appropiate promoter system in a $Notch^{ts1}$ mutant background. We reasoned that expression of *Notch* derivatives using this system would allow us to assay the function of putative signaling domains of *Notch*, specifically in the process of axon outgrowth, whereas expression with other promoters (for example, earlier in neurogenesis) would permit assay of their activities in control of cell fate.

We know (see Introduction) that the mechanism by which *Notch* controls cell identity requires that the protein translocate to the nucleus, and indeed, that a *Notch* derivative consisting only of the free intracellular domain is found primarily, if not exclusively, in the nucleus (53, 54). If *Notch* controls axon patterning by working directly in the growth cone, we would expect that a nuclear-localized *Notch* derivative would be ineffectual for rescue of the *Notch* axonal function, whereas a derivative that is tethered to the plasma membrane might still be able to promote growth cone motility and might rescue axon patterning. We therefore compared the activity of our membrane-tethered $Notch^{\Delta E}$ to that of a nuclear-localized activated Notch. First, we expressed both proteins in differentiating sensory neurons at a relatively early developmental stage. Consistent with previous results from us and from others, while both proteins induce an anti-neurogenic phenotype (ie reduce the number of neurons that develop), nuclear Notch was substantially more active than membrane-tethered Notch for controlling cell identities (ref 51; data not shown). (Others have presented evidence that $Notch^{\Delta E}$ is still capable of being cleaved and translocating to the nucleus with an efficiency that is sufficiently high to perform significant ligand-independent *Notch* signalling (16)). In contrast, while $Notch^{\Delta E}$ provided significant rescue of the axonal phentoype of a temperature-shifted $Notch^{ts1}$ mutant, nuclear *Notch* was completely without effect in this assay (Fig 5 C,D). This result shows that rescue of axon patterning requires that *Notch* be present at the plasma membrane, consistent with a direct role in growth cone function. Moreover, it validates our $Notch^{ts}$ assay system as a way to test for an axonal role of particular *Notch* domains, and it identifies the first domain that is specific for the *Notch* axonal function: the transmembrane domain.

4. Efforts to perform a genetic screen for modifiers of the *Notch/abl* interaction: Evidence that the *Notch/abl* pathway may play some role in genetic instability.

A major goal of this study (Task 4, per the amended Statement of Work of 6/9/97), was to perform a screen for genetic modifiers of the *Notch/abl* interaction as a way to identify other proteins that might act in this novel signaling pathway. We were unsuccessful in this endeavor, due to a completely unexpected roadblock: fly stocks simultaneously bearing heterozygous mutations in *Notch* and in *abl* displayed a tremendously high rate of aberrant genetic events, completely obscuring the rare mutational events that we sought to screen for. For example, recessive lethal mutations in *Drosophila* are routinely kept as heterozygotes over multiply-rearranged chromosomes, so called "balancer chromosomes" that have inversion breakpoints every few centimorgans. Since chromosomal rearrangements locally prevent genetic recombination, such trans-heterozyogous combinations are stable literally for decades. In contrast, in the *Notch/abl* genetic background we routinely observed markers recombining back and forth between, for example, testor chromosomes and their balancers, making it impossible to reliably trace markers through the crosses necessary to perform and analyze the mutagenesis experiment. Moreover, we also observed chromosome nondisjunction events at extraordinarily high frequencies -- as high as 5 - 10% of progeny even in some relatively simple crosses. Again, this is many orders of magnitude more frequent than any authentic mutational event could possibly be, and made our efforts at a genetic screen completely fruitless.

Why should the *Notch/abl* interaction produce such a high level of genetic instability? Three possibilities occurred to us. First, it could be that the level of instability is actually not so different from the wild type situation, but the poor viability of individuals that are mutant for both *Notch* and *abl* provided an extremely strong selection for events which can occur, but are very rare and usually not recovered, in a normal population. This seems to us to be extremely unlikely. We have used many multiply mutant *Drosophila* stocks in other experiments over the past 10 years, including many that carry mutations which have lethal interactions akin to that of *Notch* and *abl*, without observing this sort of apparent instability. The second possibility arises from a peculiarity of fly genetics. The nucleolar organizer is found on the X and Y chromosomes in the fly. Occassionally, lab stocks will suffer deletions that remove various portions of the organizer on the X. When this happens, the stock may be somewhat less robust than a wild type fly, particularly if it is stressed, for example, by bearing additional mutations. Since XXY *Drosophila* females are fertile, female flies with partial deletions of the X-linked nucleolar organizer will sometimes pick up a copy of the Y chromosome. This subsequently leads to high frequencies of sex chromosome nondisjunction, and sometimes increased rates of autosomal nondisjunction as well. If *Notch/abl* stocks have extra Y chromosomes that might at least explain the elevated nondisjunction frequency. Again, however, we do not find this explanation compelling. The X chromosomes in the stocks in which we have observed genetic instability come from four unrelated *Notch* mutants (*Notch* is on the X chromosome), in combination with three different balancers, and yet all these combinations display similar instability. Nonetheless, we cannot yet rule out this model unambiguously. Finally, the third possibility is that the *Notch/abl* pathway actually plays some role in maintaining genome integrity in wild type animals. Consistent with this possibility, one of the best characterized *in vivo* functions of mammalian Abl is in the DNA damage checkpoint, downstream of ATM (55). We know that *Drosophila* has genes homologous to the *ATM* and *Chk1* genes that are central to the DNA damage checkpoint in yeast and mammals, and we know, moreover, that these genes are required both for the fly's response to DNA damaging agents and for proper mitosis in the early embryo (56, 57). If *Notch* and *abl* are involved in the maintenance of genome integrity, that could help explain the instability we observed in flies bearing both mutations. Moreover, this would certainly suggest an additional reason why the mammalian homologues of these genes are important contributors to oncogenesis. We therefore set out to determine rigorously whether *Notch* and *abl* mutations lead to genetic instability.

In order to distinguish whether the apparent genetic instability of flies bearing *Notch* and *abl* mutations arises from the presence of extra Y chromosomes, particularly in XXY females, we have prepared stocks in which all X and Y chromosomes are genetically marked. Moreover, to assay *Notch/abl* flies for autosomal as well as sex chromosome nondisjunction, we have obtained testor fly stocks bearing compound autosomes that will give euploid progeny only if crossed to flies that fail to disjoin their autosomes. For these experiments we have prepared fly stocks that are doubly heterozygous for *Notch* and for *abl*, using two unrelated *Notch* alleles, combined with two different *abl* alleles. As a control for the specificity of any effect we observe, we have also generated these stocks with complementing duplications for *Notch*, or for *abl*, and we have prepared stocks that have the same *abl* mutations but with a different X chromosome mutation (*Df(1)sc*, to compare with *Df(1)N* flies). All of these stocks have now been prepared, and we are in the process of expanding the stocks so that we can perform the appropriate crosses. We expect to perform the actual experiment over the next several months.

5. Analysis of potential Abl effectors in *Notch*-dependent developmental decisions

Given the impediments to performing a genetic screen for downstream elements of the *Notch/abl* pathway, we turned our attention to considering plausible candidate genes. A significant body of work suggests that Rho subfamily small GTPases, particularly Rac and Cdc42, act as Abl effector proteins in mammals. We know that extension of all axons requires carefully controlled levels of activity of both of these GTPases (58). We wished to know, however, whether they played any part specifically in developmental processes that are *Notch*-dependent but *Su(H)*- independent, and

thus potentially in the *Notch/abl* pathway. As a first step, we exploited the neuron-glia decision of adult sense organ inner precursor cells (see above). We therefore expressed a constitutively-activated Rac (Rac^{G12V}) in cells of the sense organ lineage. We found that activation of *rac* in inner precursors produced the same phenotype as activation of *Notch*: transformation of the neuron into a second glial cell (Fig 6). This is potentially consistent with the possibility that *rac* acts in *Notch* signaling in inner precursor cells, as predicted if this developmental decision employs the *Notch/abl* pathway. For reasons we do not understand, however, expression of a dominant negative Rac (Rac^{S89L}) gives a rather more complex phenotype, apparently with extra copies of both cell types (data not shown). Additional genetic dosage and epistasis experiments will be required to determine the significance of these phenotypes, and in particular whether *rac* is acting downstream of *Notch* and *abl* in a dependent pathway.

C. Recommendations

Our recommendations for the furtherance of this project are as follows:

1. Activities of Notch protein *in vivo* (Task 1)
For the purposes of this project, further identification and characterization of wild type functions of *Notch* is not immediately necessary. The one exception is investigation of the possibility that a myogenic FGF-like activity is regulated by *Notch* signaling in the embryonic ectoderm. This should be addressed by investigating the genetic epistasis between ectodermal *Notch* and the myogenic FGF receptor, or by directly analyzing the effect of *Notch* on expression of the relevant FGF, once this gene is identified.

2. Domains of *Notch* (Task 2)
In order to characterize the mechanism of *Notch*-dependent control of axon outgrowth, the most pressing need is for a thorough deletion analysis of the *Notch* intracellular domain to complete the identification of domains required for this process *in vivo*. For this purpose, it will be best to employ the system we have established of constructing derivatives of full-length *Notch* bearing alterations in the intracellular domain, and assaying their ability to complement the axonal defects of temperature-shifted $Notch^{ts1}$ embryos.

Experiments we have performed to date suggest that the amino terminal half of the intracellular domain will be sufficient to promote axon extension when reconstructed into the context of the complete extracellular domain. We predict that the Ram23 domain will be necessary, and in particular the Disabled binding site(s). We have as yet no data on whether the ankyrin repeats will be required, but this experiment will be interesting whatever the outcome.

To focus the deletion analysis of *Notch* more precisely, it will be important to continue characterizing the biochemical basis of the Notch-Disabled interaction (Task 3 of the modified Statement of Work of 6/9/97). We are currently refining the deletion analysis of the Ram23 domain by making finer deletions (20-35 amino acids). These will be assayed individually both for binding to the Disabled PTB domain and to Δ-PTB Disabled. The minimal mutation that prevents binding of Disabled to Notch should then be reconstructed into the full-length *Notch* gene and assayed for *Notch* axonal function as above. It will also be important to use a deletion analysis to localize the second putative Notch-binding domain of Disabled. Finally, we have used site-directed mutagenesis to make a mutation in the Ram23 domain of *Drosophila Notch* that is identical to a mutation which has been shown to abolish the binding of mouse Notch1 to mouse Su(H) (59). Assuming that this mutation does not affect binding of Disabled (which we are currently testing) we would predict that this mutation will impair *Notch*-dependent control of cell fate without affecting *Notch*-dependent growth cone motility.

We had originally proposed a different system to dissect the domain structure of *Notch*, involving analysis of the dominant effects of activated *Notch* derivatives. However, as discussed in detail in our Annual Report of 10/96 (in the section "Activated Notch and ectopic axon outgrowth"), this approach proved impractical. We therefore do not recommend that such an experiment be undertaken. The time required to thoroughly rule out our original plan, together with the need to develop and validate an alternate assay, significantly delayed this aspect of our study. The original proposal also called for performing a deletion analysis of the mouse *Notch1* gene, using *Drosophila* as the assay system. Feedback we received from the reviewers of this proposal, as well as feedback from numerous other workers in the field, was essentially unanimous that an analysis of the mouse gene in this heterologous system was potentially fraught with artifacts, and that our time would be better spent concentrating on the analysis of the fly gene. We now concur with this advice, and do not recommend a deletion analysis of the mouse gene in the fly system. Rather, once we know more about the domain structure of the fly gene it should be possible to design experiments that can be done directly in a mouse assay system to determine the generalizability of our findings.

3. Genetics of the *Notch/abl* pathway

a. Genetic screen for modifiers of the axonal phenotypes of activated *Notch* (Tasks 3 and 4 of the original proposal)

As referred to under Recommendation 2 above, this approach proved impractical and was replaced with analysis of the *Notch/abl* pathway under a modified Statement of Work (6/9/97)

b. *Notch*, *abl* and genetic instability

If the *Notch/abl* pathway indeed contributes to genome integrity, this would significantly affect how we think about the roles of *Notch* and *abl* in cancer. It is therefore critical to complete the experiment of assaying chromosome stability in various *Notch/abl* mutant backgrounds. The finding that the instability of *Notch/abl* flies arises from supernumerary Y chromosomes would suggest that the instability we have observed is a peculiarity of fly biology, and not of general interest. It would also suggest that with appropriately marked strains it may be possible to execute our original scheme for continued genetic analysis of the *Notch/abl* pathway. The finding that this instability reflects an activity intrinsic to the *Notch/abl* pathway would open up a new area for study, and potentially one of great translational significance.

c. Genetic screens for modifiers of the *Notch/abl* interaction (Task 4 of the modified Statement of Work)

Unless conditions can be found to alleviate the apparent genetic instability of stocks simultaneously bearing mutations in *Notch* and *abl*, or to identify quickly those progeny from each cross that reflect illegitimate genetic events, the prospects for doing a genetic modifier screen are poor. In contrast, should candidate genes come to light (see below), it is possible to arrange chromosomes to do tests of those candidates in ways that permit aberrant events to be recognized and discounted. The best way to begin identifying candidate genes will be to characterize the minimal *Notch* derivative that can perform the axonal function of *Notch* and find the proteins and genes that interact with the *Notch* domains so identified.

d. *abl*, *rac* and sense organ differentiation

The finding that *rac* can effect the *Notch*-dependent, *Su(H)*-independent decision between neuronal and glial fate of inner precursor cells potentially affords an alternate approach to identifying genes in the *Notch/abl* pathway. The key experiments, clearly, are first to determine whether this cell fate decision involves *abl*, or alternatively whether the participation of *rac* reflects yet another previously unrecognized *Notch* signaling pathway. The simplest way to discriminate between these options is by assaying sense organ cells in *abl* mutant pupae, and by determining whether an *abl* mutation sensitizes sense organ cells to reduced levels of *Notch* signaling. In addition, it will be crucial to use standard genetic epistasis experiments to determine whether these genes form a single dependent pathway or parallel pathways.

7. CONCLUSIONS AND IMPLICATIONS

The major goals of our study were successfully achieved: we demonstrated that *Notch* directly controls axon patterning by a mechanism that is genetically distinct from its control of cell fate; established a straightforward assay system for testing which domains of *Notch* were active specifically in that pathway, and found that the *Notch* transmembrane domain was one such domain; showed that the *abl/disabled* signaling pathway is the pathway that cooperates with *Notch* in its direct control of axon outgrowth; obtained suggestive evidence that Rho family GTPases were plausible candidates for downstream effectors of *Notch* function, possibly as part of this novel *Notch/abl* pathway; and verified experimentally that, as speculated by others, *Notch* inhibits differentiation *in vivo* in a way that potentially contribute to its activity as an oncogene.

The data summarized above have a number of implications for our thinking about *Notch* in cancer, and specifically in breast cancer.

A. These data support the original notion that the activity of *Notch* as an oncogene may well reflect the ability of this single protein to control, independently, both the identity and the motility of cells. We already know from the work on mouse *int3* that *Notch* can be associated with breast cancer; it might well be instructive to investigate directly whether the *Notch* pathway is dysregulated in breast cancer arising from other causes.

B. Given that *Notch* and *abl* are both ubiquitously expressed proteins that are conserved throughout metazoan evolution, it seems likely that the *Notch/abl* pathway we have discovered is equally widespread. Given further that both of these genes are established human oncogenes, it is certainly plausible that the *Notch/abl* pathway will be found to play a significant role in human cancer. It therefore seems self-evident that it would be worthwhile to investigate *abl* activity in those malignancies in which *Notch* has been implicated, including breast cancer, as well as to investigate *Notch* activity in *abl*-associated malignancies, such as chronic myelogenous leukemia.

C. The evidence that *Notch* in epithelial cells may control the differentiation of an underlying mesenchyme by regulating FGF expression is potentially of direct relevance to breast cancer. It suggests that it may be worthwhile to assay for alterations in FGF expression in a mammalian model of *Notch*-induced mammary carcinogenesis, such as mice bearing the *MMTV-int3* transgene, and to investigate FGF levels and *Notch* signaling in patient tumor tissue.

D. From the viewpoint of understanding axon patterning, the most important implication of this work is that the *Notch/abl*-dependent regulation of growth cone motility along CNS interface glia and PNS peritracheal cells now provides one of the only guidance decisions in any system for which a receptor that is genetically known to be required for that guidance decision *in vivo* has been linked directly to axonal signaling proteins that are also known genetically to be required for the growth and guidance of those same axons *in vivo*. As such, this becomes one of the best characterized systems yet described for analysis of the mechanism of axon growth and guidance.

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9. APPENDICES

Figure Legends:

Figure 1. *Notch$^{\Delta E}$* transforms cell identities in developing adult sense organs

A: A schematic indicating the lineage by which a sense organ precursor (also called the "mother cell") undergoes two rounds of cell division to give rise to the four cells of the mature sense organ.

B-D: Phenotypes observed upon expression of *Notch$^{\Delta E}$* in cells of the sense organ lineage, driven by the GAL4 line 109-68. This GAL4 line causes expression of the *Notch$^{\Delta E}$* effector transgene in the sensory mother cell and its progeny, but does not come on early enough to affect the initial segregation of the mother cell itself. Note also that expression using the GAL4 system is typically 'patchy'; not all sense organs, or all cells of any given organ, will necessarily express the effector transgene. B and C show Nomarski images of the cuticle of adult flies expressing *Notch$^{\Delta E}$* ; D shows an expressing pupa ~24 hours after pupariation, stained with mAb MR1a (to label glial cell nuclei) and mAb 22C10 (to label neuronal cell cytoplasm) and visualized by peroxidase immunocytochemistry.
B: Open arrow indicates a morphologically wild type sense organ (microchaete). Note the bristle, and the round socket where it attaches to the cuticle. Closed arrows indicate a transformed sense organ; note the absence of a bristle, and in its place, the appearance of two socket-like structures.
C: Arrow indicates a sense organ (macrochaete) in which four socket-like structures are observed, but no bristle.
D. Open arrow indicates an apparently wild type sense organ; note the small round (dark) glial nucleus, next to the (lighter) elongated neuron with an axon projecting to the right. Closed arrows indicate a sense organ in which the nuclei of both inner progeny cells label with mAb MR1a, but the cytoplasm of neither labels with 22C10, suggesting that both cells have differentiated as glial cells.

Figure 2. Separation of the axonal and cell fate functions of *Notch.*

Notch^{ts1} embryos that did (B,D) or did not (A,C) bear *elav-GAL4* and *UAS-Notch* transgenes were collected at permissive temperature, shifted to restrictive temperature in mid-embryogenesis and fixed for immunocytochemistry. (A,B) Ventral views of st 16 embryos stained with anti-Fasciclin 2. Arrows indicate positions of longitudinal axon tracts (absent in A, but restored in B). (C,D) Embryos stained with anti-Odd. Note that expression of wild type Notch by this protocol does not rescue cell identities: six cells label with anti-Odd in each segment of both embryos, instead of the wild type pattern of four cells. For comparison, Panel E shows the Odd staining pattern of a wild type embryo. Each panel shows three segments of the CNS.

Figure 3. Notch protein is found in growth cones.

Drosophila embryos that express a kinesin-β-galactosidase fusion in neurons (KZ 636) were mechanically dissociated, cells were plated on clean glass coverslips and allowed to develop overnight. Cells were then fixed and double-stained with anti-β-galactosidase (FITC, green) and anti-NOTCH (Texas Red). FITC signal is shown in Panel A, Texas Red signal in Panel B; Panel C show the overlap of the fluorescence signals. Growth cone is indicated with a white arrow.

Figure 4. *in vitro* binding of soluble Disabled protein to Notch.

Beads bearing His6-tagged fusions of either the Ram23 domain or the ankyrin repeats of Notch were incubated with *in vitro*-translated Disabled protein or derivatives of Disabled protein. Beads were then washed, and the bound proteins analyzed by SDS PAGE and autoradiography.

A: Full length Disabled (arrow) associates with Ram23 beads but not with beads bearing the Notch ankyrin repeats. Lane at the left shows 2.5% of the input protein to the binding reaction; positions of molecular weight markers are indicated to the right.

B: The Disabled PTB domain binds specifically to the Ram23 domain of Notch. Labelling as in panel A. For reasons we do not understand, in this experiment the isolated PTB domain ran as a close-spaced doublet.

C: A Disabled derivative lacking the PTB domain nonetheless associates with Notch. Experiment performed and labelled as in A, above.

Figure 5. Membrane-tethered *Notch*, but not nuclear *Notch*, can perform the *Notch* axonal function

A constitutively activated derivative of *Notch* (consisting only of the complete intracellular domain; top panel), or $Notch^{\Delta E}$ (retaining a signal sequence and transmembrane domain; bottom panel) were expressed in the $Notch^{ts1}$ mutant background using *elav-GAL4*, precisely as in the experiment of Figure 2. Stage 16 embryos were fixed and stained with anti-Fasciclin 2 to visualize CNS longitudinal axons.

Top: Longitudinal axons of temperature-shifted $Notch^{ts1}$ mutant embryos are not rescued by expression of nuclear activated Notch: arrow indicates position where longitudinal axons should be seen.

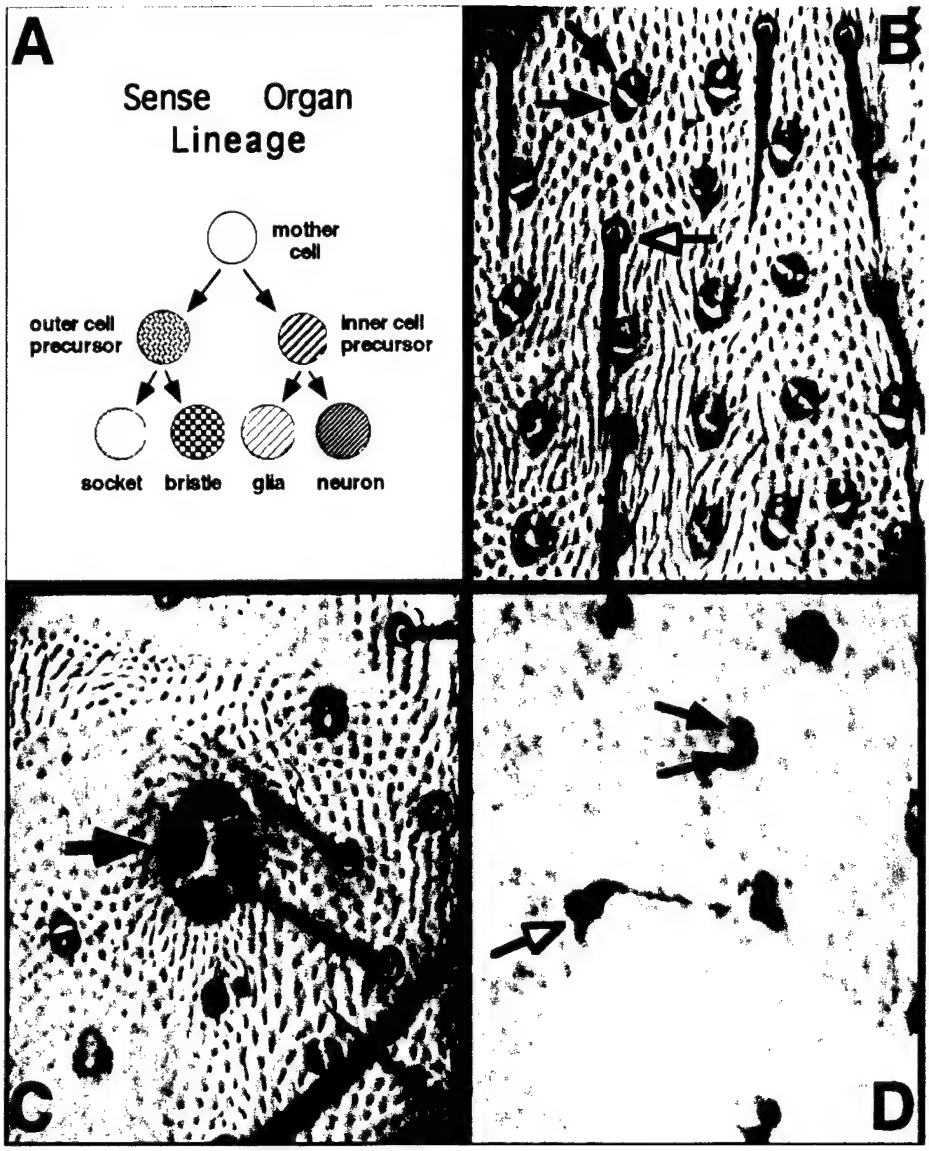
Bottom: $Notch^{\Delta E}$ partly rescues longitudinal axons of $Notch^{ts1}$ embryos (arrow). The observation that a *Notch* derivative lacking all extracellular sequences can partly rescue *patterning* implies that there must be other proteins that also contribute spatial information to *Notch*-dependent growth cones; the fact that the rescue is so incomplete (compare Figure 2B) may suggest that *Notch* contributes *some* spatial information.

Figure 6. Activation of *rac* phenocopies activation of *Notch* in adult sense organ development

Activated *Drosophila rac* (rac^{G12V}) was expressed using GAL4 line 109-68, just as for the expression of $Notch^{\Delta E}$ in the experiment of Figure 1. Pupae were collected 24-27 hours after pupariation, fixed, and stained with mAb MR1a (to label glial nuclei; Panel A) or anti-Elav (to label neuronal nuclei; Panel B) and visualized with peroxidast immunocytochemistry.

A: Notum of a pupa 24 -27 hours after pupariation; open arrow indicates an apparently wild type sense organ with only a single glial cell labelled; closed arrows indicate a sense organ in which both cells have differentiated as glia. Note that a substantial fraction of the sense organs in this notum appear to contain multiple glial cells.

B: View of a pupal notum equivalent to that shown in A, but stained with anti-Elav to label neuronal nuclei. Very few of the sense organs in this notum appear to contain any neurons; open arrow indicates one sense organ in which a neuron did differentiate.


A
Sense Organ
Lineage
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cell
outer cell
precursor
inner cell
precursor
socket
bristle
glia
neuron
B
C
D

Figure 1

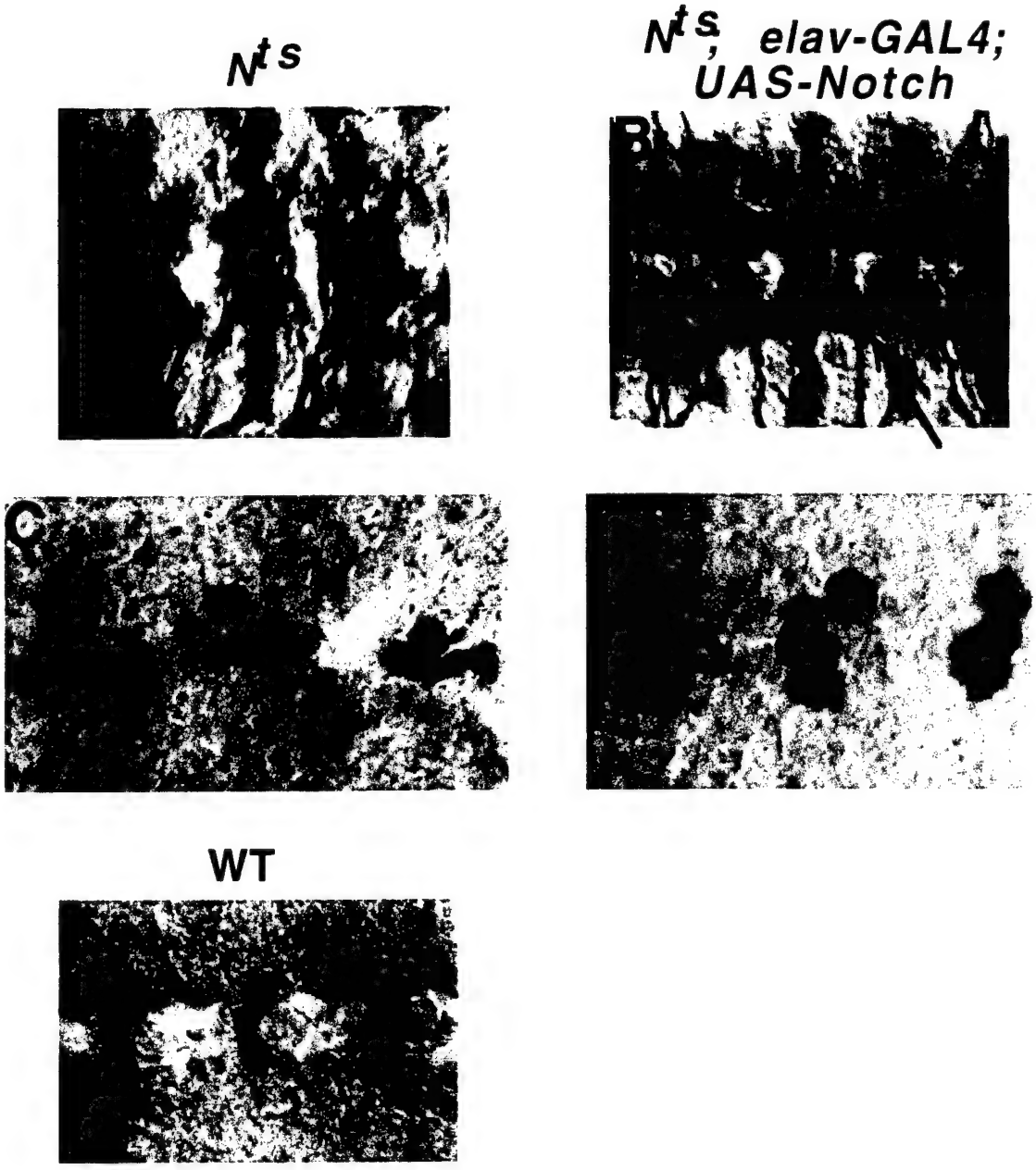


Figure 2

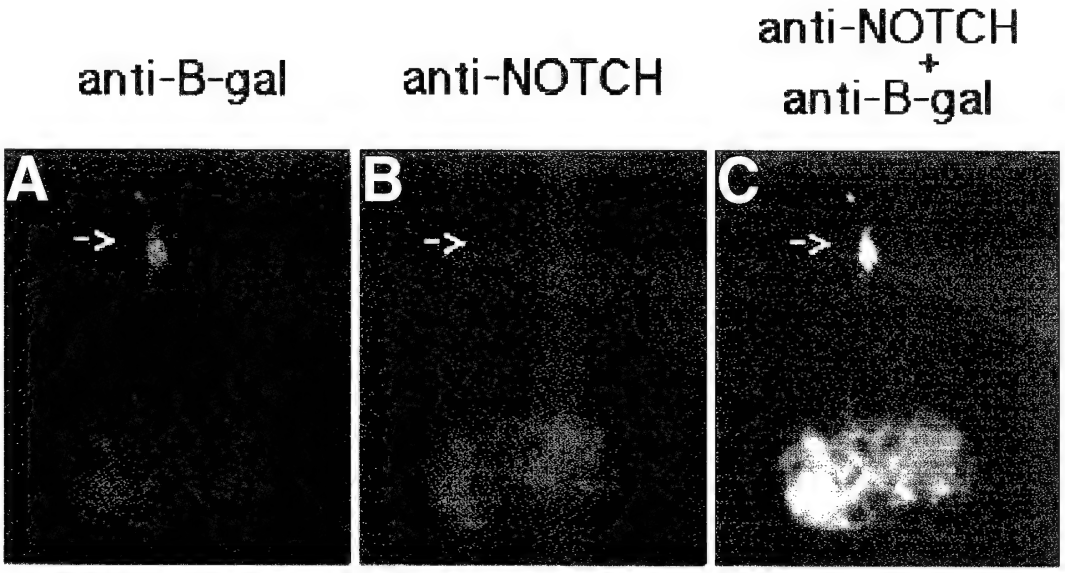
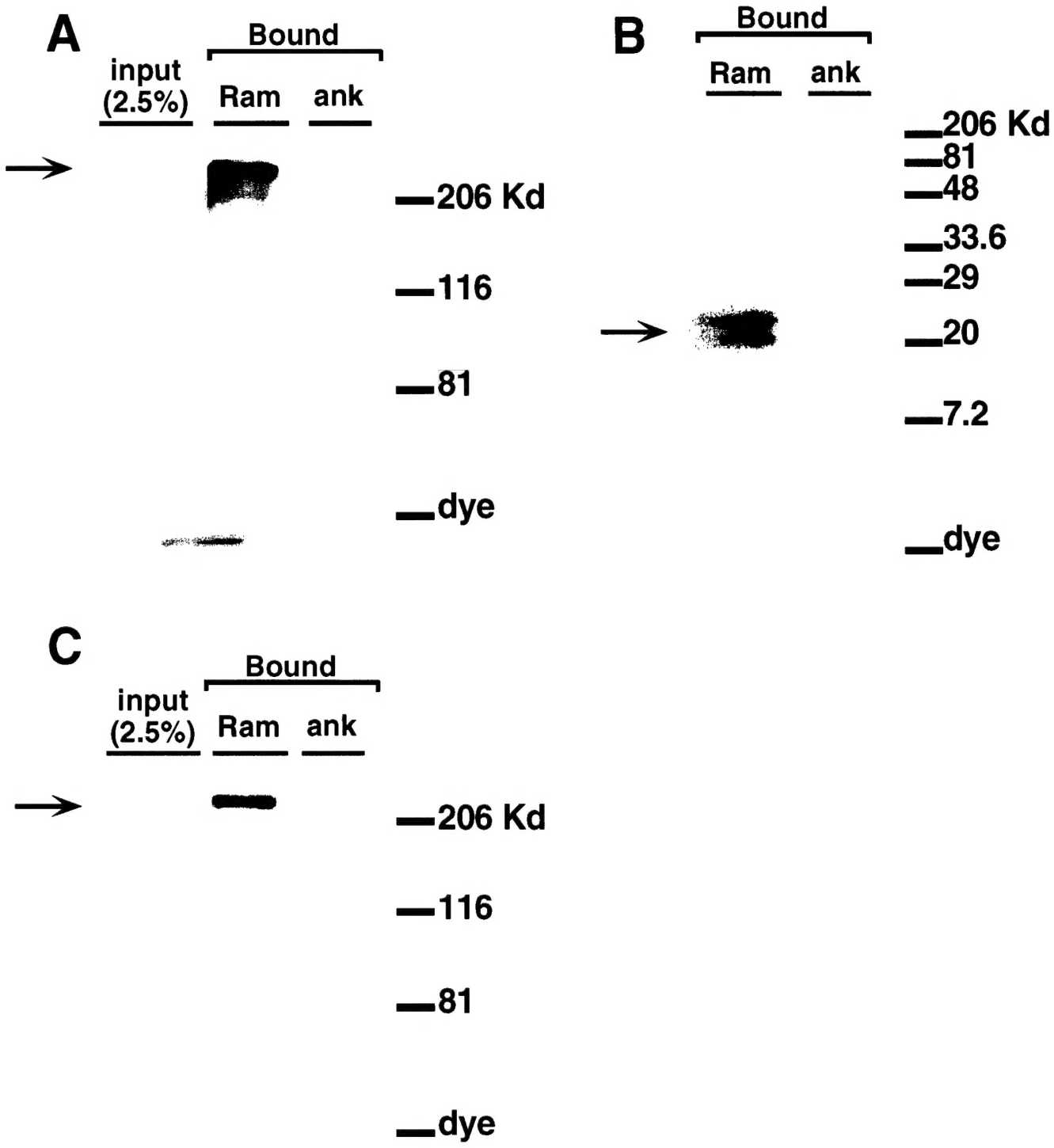


Figure 3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Bound
input
(2.5%)
Ram
ank
206 Kd
116
81
dye
B
Bound
Ram
ank
206 Kd
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48
33.6
29
20
7.2
dye
C
Bound
input
(2.5%)
Ram
ank
206 Kd
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dye

Figure 4

N^{ts} with nuclear Notch expressed in postmitotic neurons

N^{ts} with membrane-tethered Notch expressed in postmitotic neurons

Figure 5

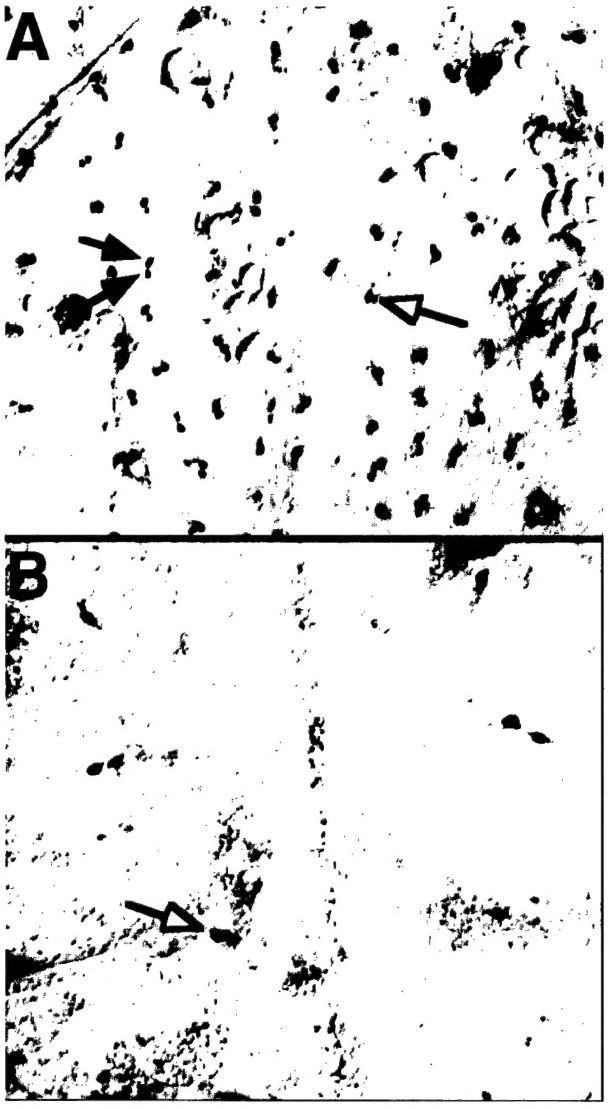

Figure 6

11. BIBLIOGRAPHY AND PERSONNEL

A. Published Papers:

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B. Published Meeting Abstracts:

Drosophila Genetics Research Conference (1996) *Control of axon guidance by the D. melanogaster gene lola.* K. Madden (presenting) and E. Giniger.

DOD Breast Cancer Meeting (1997) *Notch and the control of cell fate and morphology. Activities and signaling pathways.* E. Giniger (presenting), L. Connell-Crowley, D. Crowner, S. Fuerstenberg and K. Tietje

(Note: as specified in the Detailed Cost Estimate Justification, most of the meetings attended with support from this grant were Gordon Research Conferences, for which no formal abstract is submitted)

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